

# Direct estimates of the contribution of viral lysis and microzooplankton grazing to the decline of a *Micromonas* spp. population

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**ABSTRACT:** During a mesocosm study in Raunefjorden, Norway, a *Micromonas* spp. population, initially showing exponential net growth, dramatically declined after Day 4 of the experiment. Using a modification of the dilution approach originally developed to quantify grazing by microzooplankton on phytoplankton, it was possible to partition the mortality of *Micromonas* spp. between grazing and viral lysis on Days 5, 6 and 7 during the population decline. Parallel dilution experiments were carried out in which 0.2 µm- and 10 kDa-filtered water was used as the diluents. In this way, gradients of grazing pressure (0.2 µm series) and grazing pressure + viral concentration (10 kDa series) were produced. Model 1 linear regression of the fraction of whole water versus the apparent growth rate of chlorophyll *a* and *Micromonas* spp. provided an estimate of mortality in the 0.2 µm and 10 kDa dilution series. On Days 5, 6 and 7, the slopes of the linear regressions of 0.2 µm and 10 kDa dilution series were significantly different at  $p = 0.083$ ,  $0.001$  and  $0.093$  respectively. From the differences in slope between the series, estimates of viral mortality amounted to a turnover rate of the *Micromonas* spp. standing stocks of 10, 25 and 9% d<sup>-1</sup>. This compares to a turnover rate by the microzooplankton of 48, 26 and 23% d<sup>-1</sup>. On all 3 d the combined viral lysis and grazing mortality exceeded estimates of the potential production of *Micromonas* spp., in part accounting for the population decline. This study demonstrates that the dilution approach can be adapted to directly determine virus-induced mortality rates of specific phytoplankton. However, further work is required to determine how the specificity of viral infection and variety of viral infection cycles affect the results of this modified dilution approach when applied to other phytoplankton taxa and communities.

**KEY WORDS:** Viral lysis · Microzooplankton grazing · *Micromonas* spp. · Dilution technique

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## INTRODUCTION

Grazing and viral lysis have been identified as significant sources of mortality in marine bacterioplankton (Fuhrman & Noble 1995, Weinbauer & Peduzzi 1995) and eukaryotic phytoplankton (Banse 1992, Cottrell & Suttle 1995a). Whether cells are grazed or lysed has different ecological and biogeochemical conse-

quences, since there are implications for the flow of matter and energy through the microbial web (Fuhrman 1999, Wilhelm & Suttle 1999). Production grazed may be passed to higher trophic levels and eventually lost from the euphotic zone via sinking, whereas viral lysis has the net effect of regenerating nutrients and recycling carbon (Bratbak et al. 1994, Gobler et al. 1997, Fuhrman 1999). Ecologically the different causes of mortality affect the size structure, diversity and distribution of marine microbial commu-

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nities. Although grazers have been shown to exhibit a preference for certain prey (Epstein & Shiaris 1992), this form of selection pressure is minimal compared to that exerted by the highly specific nature of viral infection (Hennes et al. 1995, Fuhrman 1999, van Hannen et al. 1999). Of additional ecological significance is the ability of viruses to transfer genetic material (Chuirra 1997).

Previous studies, which have examined the degree of mortality caused by grazing or viral lysis, have focused on bacteria (Fuhrman & Noble 1995, Weinbauer & Peduzzi 1995, Steward et al. 1996, Weinbauer & Holfe 1998, Guixa-Boixereu et al. 1999, Tuomi & Kuuppo 1999, Pedrós-Alió et al. 2000). Collectively these studies indicate that the importance of grazing and viral lysis varies for different environments, host organisms, and seasons. No single technique has been applied that simultaneously quantifies both grazing- and viral-induced mortality. A dilution approach introduced by Landry & Hassett (1982) is now routinely used to quantify grazing by microzooplankton on phytoplankton. The present study describes a modification of this approach, which provides concurrent estimates of viral- and microzooplankton grazing-induced mortality of a specific eukaryotic phytoplankton.

In standard dilution experiments, the net growth rate of phytoplankton ( $k$ ) in incubations is considered to be a product of the instantaneous growth ( $\mu$ ) and mortality ( $m$ ) of phytoplankton due to grazing ( $m_g$ ) (Fig. 1). It

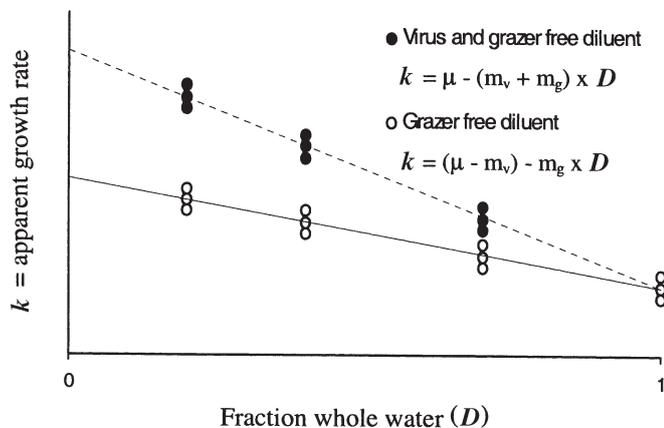


Fig. 1. Idealised dilution plots and analysis when filtered water containing viruses (standard protocol used to determine grazing rates) and viral-free water are used to dilute whole water in the incubations that make up each dilution series. Higher growth rates are expected in the latter due to the removal of an additional cause of mortality ( $k$  = net growth rate of phytoplankton,  $\mu$  = instantaneous growth rate of phytoplankton,  $m_g$  = mortality due to microzooplankton grazing,  $m_v$  = mortality due to viral lysis,  $D$  = fraction of whole water)

is assumed that  $\mu$  remains constant with respect to the level of dilution ( $D$ ) whilst  $m_g$  is considered to be density dependent. The viral component of mortality is ignored in standard analyses. Diluent produced in standard dilution experiments is generally filtered through  $\geq 0.2 \mu\text{m}$  pore size filters through which almost all free viruses are likely to pass. As viral infection rates are density dependent, it can be assumed that specific viral mortality of phytoplankton ( $m_v$ ) is constant with respect to  $D$  and therefore that estimates of  $\mu$ , the  $y$ -axis intercept, include a viral mortality component (Fig. 1). Alternatively, if the diluent utilised is viral free, the net phytoplankton growth rate at each level of dilution will be a product of the instantaneous growth rate and density dependent viral plus grazing mortality components (Fig. 1). A direct estimate of viral mortality of the phytoplankton can be obtained from the difference in the relationship between  $k$  and  $D$  in incubations diluted with viral-free and viral-containing diluent ( $m_v = (m_v + m_g) - m_g$ ).

The present study formed part of a larger mesocosm experiment which examined the progression and fate of *Emiliania huxleyi* populations under differing nutrient regimes (Jacquet et al. 2002) and also with regard to the production of dimethyl sulphide. During the early stages of the mesocosm experiment we successfully applied the modified dilution approach to quantify the relative impact of viral- and microzooplankton-induced mortality of a *Micromonas* spp. population.

## MATERIALS AND METHODS

**Mesocosm experiment.** The mesocosm experiment was carried out from June 5 to June 25, 2000, at the Marine Biological Station (Espeland) adjacent to Raunefjorden, 20 km south of Bergen, western Norway. The mesocosms used were situated along the southern side of a raft located approximately 200 m offshore. The mesocosm bags were secured to floating frames moored to the raft. The enclosures were approximately 4.5 m deep, 2 m in diameter, had a volume of  $11 \text{ m}^3$  and were made from 0.15 mm thick polyethylene which transmits 90% of photosynthetically active radiation. They were open to the air and the water was kept homogeneous by an air lift system. (For details see Egge & Aksnes 1992). The mesocosm bags were filled on June 5 by pumping unfiltered fjord water from a depth of 2 m.

Inorganic nutrient enrichment commenced on June 6 and continued daily at ca. 11:00 h. Initially all bags were supplied with  $1.5 \mu\text{M}$  nitrate ( $\text{NaNO}_3$ ) and  $0.1 \mu\text{M}$  phosphate ( $\text{K}_2\text{HPO}_4$ ). From 12 June the nutrient regime was altered to manipulate Bag 2 into phosphate depleted conditions by daily additions of  $1.5 \mu\text{M}$

N-NO<sub>3</sub> and 0.02 µM P-PO<sub>4</sub>, and Bag 3 into nitrate depleted conditions by daily additions of 0.5 µM N-NO<sub>3</sub> and 0.1 µM P-PO<sub>4</sub>. Nutrient additions to Bag 1 remained constant throughout the study.

Nutrients, temperature, salinity, oxygen concentration and chlorophyll *a* concentration were measured daily, as were the composition and abundance of phytoplankton in the 3 bags. Analyses of nutrient concentrations were performed using an autoanalyser (Føyn et al. 1981) equipped with autosampling, detection and computing units from SANplus Segmented Flow Analyser (Skalar Analytic) according to a method adapted from Strickland & Parsons (1972). Temperature, salinity, and oxygen concentration measurements were made using a multiparameter water quality monitor OTS, Isi Model 85. Samples for chlorophyll *a* analysis (Parsons et al. 1984) were filtered at 5 mm Hg onto GF/F glass fibre filters, which were then wrapped in aluminium foil and snap frozen in liquid nitrogen. Samples were stored at -20°C in darkness until they could be analysed. Filters were then extracted in cold 90% acetone in darkness for 24 h prior to analysis on a Turner Design Model 10-AU fluorometer. Triplicate measurements were made before and after the addition of HCL to allow for the separate determination of chlorophyll *a* and phaeophytin. Phytoplankton abundance and composition were determined by analysis of fresh samples on a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm and with standard filter set-up. Fresh samples were analysed at high flow rate (~70 µl min<sup>-1</sup>) and were discriminated on the basis of side scatter and chlorophyll fluorescence. Files were analysed using CYTOWIN (available at <http://www.sb-roscoff.fr/Phyto/cyto.html#cytowin>).

**Dilution experiments.** Parallel dilution experiments were set up at ca. 6:00 h on 10, 11 and 12 June using water extracted from Bags 1, 2 and 3 respectively. Firstly, a standard dilution experiment (0.2 µm diluent) was set up according to the original protocol of Landry & Hassett (1982). Water was collected from the enclosure by submerging a 20 l polyethylene carboy (Nalgene®) and allowing it to fill gently. The particle-free fraction was prepared first by gravity filtration through a 0.2 µm filter (Gelman Supor-capsule filters) into a second carboy. From this it was transferred into a further 4 carboys in the appropriate volumes in order to create 6 l in each at dilutions of 20, 40, 70 and 100% of whole water. Freshly extracted mesocosm water was added gently by siphoning through a 200 µm mesh to remove mesoplankton. For each level of dilution, triplicate, clear, 1 l polycarbonate incubation bottles (Nalgene®) were filled again by siphoning. An additional carboy was also filled from which *T*<sub>0</sub> (time zero) mea-

surements were taken. Care was taken to minimise any air trapped in the incubation bottles and immediately after their preparation they were incubated for 24 h in a large plastic bath, attached to the southern side of the raft and suspended in the fjord water. Following incubation the bottles were removed and placed in darkness at ambient temperature prior to analysis. A parallel dilution series containing a gradient of viral concentration in addition to grazing pressure was established in the same way, with the addition of a filtration step. After gravity filtration through a 0.2 µm capsule filter, the diluent was filtered by tangential flow filtration through a 10 kDa filter. All tubing, carboys and incubation bottles were acid-washed in 5% HCl, rigorously rinsed with distilled water and then with filtered seawater between each use. Furthermore, sequential rinses of 10% HCl, distilled water and finally 8 l of filtered seawater was passed through the 10 kDa filtration system between each use. Vinyl gloves were worn whenever handling water. At *T*<sub>0</sub> and *T*<sub>24</sub>, samples were taken for chlorophyll *a* analysis, determination of phytoplankton composition and abundance. Chlorophyll *a* and phytoplankton composition and abundance analysis was performed as detailed in the methods above.

In order to test the viral concentration at each level of dilution, viruses were enumerated by analytical flow cytometry (AFC). *T*<sub>0</sub> samples for flow cytometric viral enumeration were fixed for 12 h at 4°C in 0.5% (final concentration) glutaraldehyde and frozen in liquid nitrogen. Viral counts were conducted using a Becton Dickinson FACSort flow cytometer equipped with an air-cooled laser providing 15 mW at 488 nm with a standard filter set-up. Fixed, frozen samples were defrosted at room temperature and diluted 10-fold in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, pre-filtered through a 50 kDa VivaFlow 50 'flip-flow' system [Sartorius] then autoclaved) containing 0.1% (w/v) Triton X-100. Diluted samples were heated at 65°C for 15 min in the dark in the presence of SYBR Green I (Molecular Probes) at a final concentration of 10<sup>-4</sup> of the commercial solution. Samples were analysed by AFC for 2 min at a delivery rate of 12 µl min<sup>-1</sup>. The discriminator was set to green fluorescence and the detection threshold was adjusted to cut out most of the instrument noise from the blank (10 kDa-filtered seawater diluted 10-fold with TE buffer containing 0.1% Triton X-100 and SYBR Green I). Measurements of side scatter and green fluorescence were made using LYSYSTM II software (Becton Dickinson) with log amplification on a 4-decade scale. Data analysis was carried out using WinMDI 2.8 software (available at <http://facs.scripps.edu>). Dot-plots of side-scatter versus green fluorescence were used to identify different virus groups (see 'Results').

## RESULTS

## Environmental conditions in the mesocosm bags

Similar environmental conditions occurred in the mesocosm bags. Seawater temperature in the 3 bags

increased from an average of 11.9°C on 6 June to 13.1°C on 10 June and then dropped again to initial values by 14 June (Fig. 2A). Salinity decreased slightly over the 8 d from an average of 29.9 to 29.7 ppt (Fig. 2B). Light intensity at 1 m depth, measured in one of the bags, increased up until 10 June

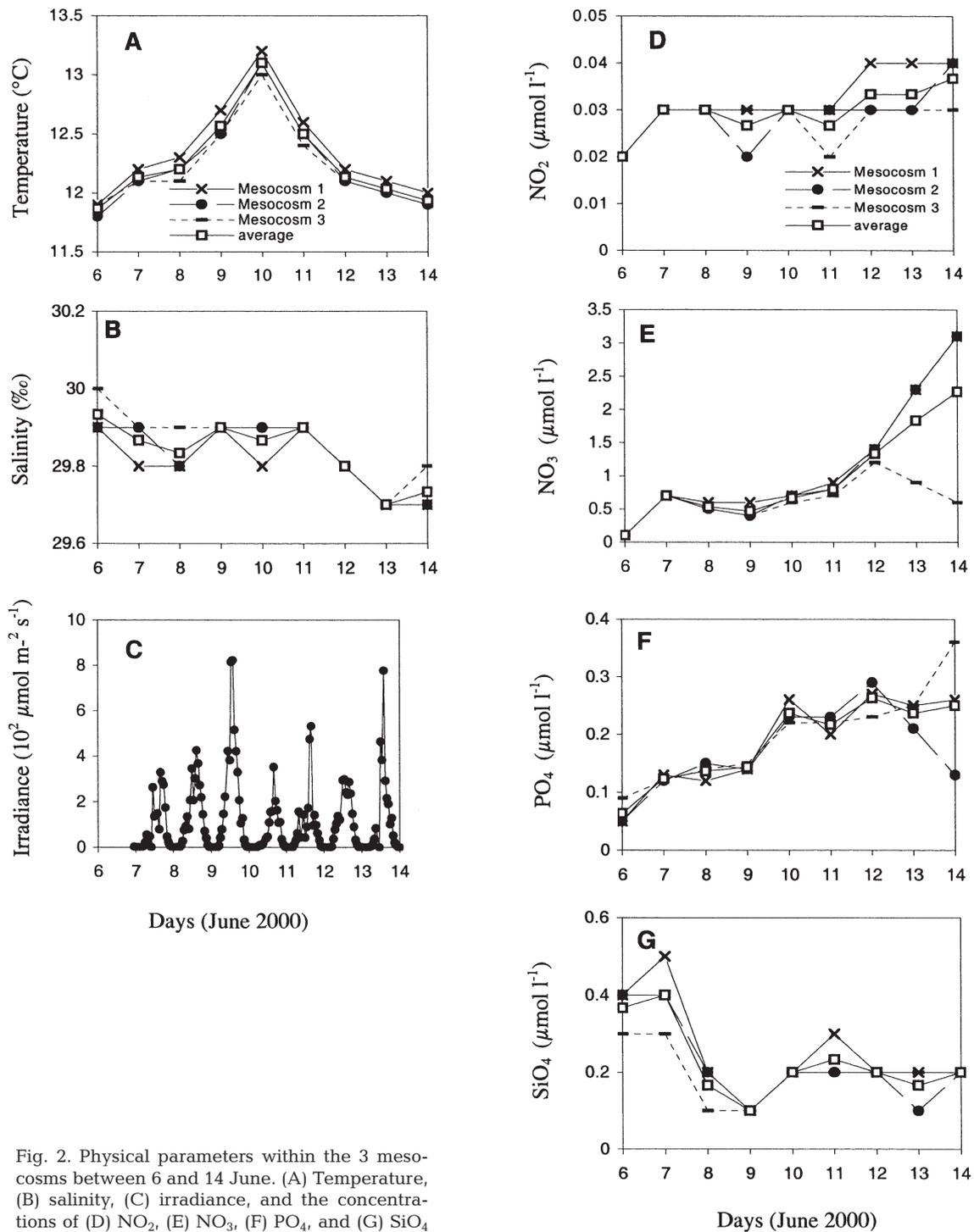


Fig. 2. Physical parameters within the 3 mesocosms between 6 and 14 June. (A) Temperature, (B) salinity, (C) irradiance, and the concentrations of (D)  $\text{NO}_2$ , (E)  $\text{NO}_3$ , (F)  $\text{PO}_4$ , and (G)  $\text{SiO}_4$

and returned to relatively low levels on the following days (Fig. 2C).

Nutrient concentrations showed similar trends in the 3 bags (Fig. 2D–G). With the daily addition of nutrients, nitrate and phosphate concentrations gradually increased up until 12 June to 1.3 and 0.26  $\mu\text{mol l}^{-1}$ , respectively. Thereafter, the daily addition of nutrients differed between the bags and this was reflected in increasing nitrate concentrations in Bags 1 and 2 and a decrease in phosphate concentration in Bag 2. Silicate showed an initial decline in concentration in all 3 bags from an average of 0.37  $\mu\text{mol l}^{-1}$  and then remained relatively stable at approximately 0.2  $\mu\text{mol l}^{-1}$  over the following 6 d. As the nutrient concentrations and dynamics in the 3 bags were similar, they were considered replicates in the present study.

### Development of the phytoplankton communities

The picoeukaryote population enumerated by AFC comprised mainly *Micromonas* spp. The identification of the population was based on characteristic AFC signatures of right angle light scatter and red and orange fluorescence observed in culture and field samples (Larsen et al. 2001). This was confirmed by sequence analysis of 18S ribosomal RNA genes, cloned from DNA extracted from mesocosm cellular concentrates, which revealed homology with known *Micromonas pusilla* 18S rDNA sequences (T. Castberg pers. comm.). Their abundance showed similar trends in the 3 bags (Fig. 3). The initial environmental conditions in the mesocosms stimulated rapid growth of the *Micromonas* spp. populations. Initial abundance averaged  $1.89 \times 10^4$  cells  $\text{ml}^{-1}$  in the 3 bags and was similar to that in the fjord. During the initial 4 d of the study, abundance increased to an average of  $8.84 \times 10^4$  cells  $\text{ml}^{-1}$  by 10 June. This represented a specific net growth rate of  $0.38 \text{ d}^{-1}$  in the 3 bags over the 4 d period. Instantaneous growth rates determined in standard dilution experiments (0.2  $\mu\text{m}$  diluent) on 7 and 8 June were 0.44 and  $1.05 \text{ d}^{-1}$ , respectively (data not shown). The populations then showed a dramatic decline over the following days to reach a minimum by 17 June of  $0.05 \times 10^4$  cells  $\text{ml}^{-1}$ . By contrast, in the fjord waters the *Micromonas* spp. population remained relatively stable at approximately  $2.00 \times 10^4$  cells  $\text{ml}^{-1}$  throughout the 8 d.

Chlorophyll *a* concentrations initially averaged  $1.04 \mu\text{g l}^{-1}$  in the 3 bags and increased at similar rates to an average of  $4.06 \mu\text{g l}^{-1}$  by 11 June. This was equivalent to a specific net growth rate of  $0.26 \text{ d}^{-1}$  over the 5 d. In standard dilution experiments, instantaneous chlorophyll *a* growth rates of 0.61 and  $0.37 \text{ d}^{-1}$  were determined on 7 and 8 June (data not shown). Concen-

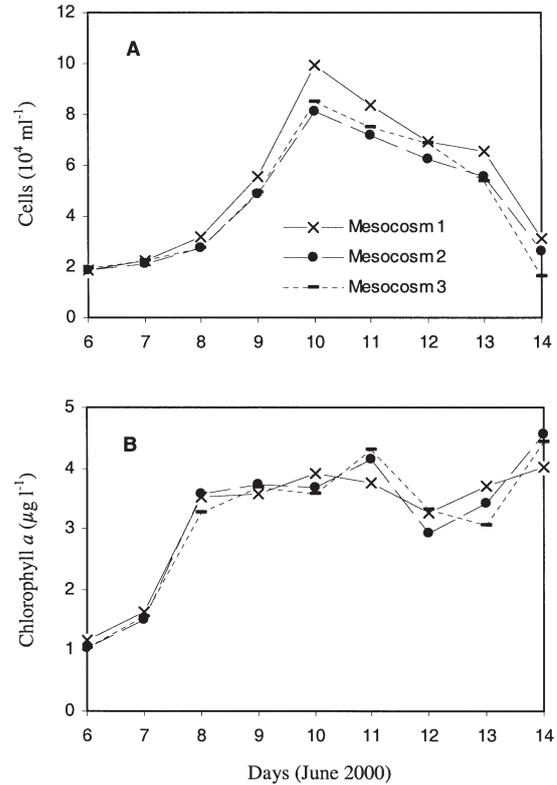


Fig. 3. Concentrations of (A) *Micromonas* spp. and (B) chlorophyll *a* between 6 and 14 June in the 3 mesocosm bags

trations then declined slightly, possibly due to the decreasing *Micromonas* spp. abundance. Based on a cell-specific chlorophyll *a* content of 0.02 pg for *Micromonas pusilla* (Montagnes et al. 1994), the *Micromonas* spp. populations in the 3 bags may have contributed as much as 48% (average of 3 bags) of the total chlorophyll *a* on 10 June. The chlorophyll *a* concentrations began to increase again after 13 June as different components of the phytoplankton community proliferated.

### Viral abundance and temporal change

The 2 populations of viral-like particles (VLPs) identified and enumerated by AFC are illustrated in Fig. 4. Population 1 (VLP1) exhibited lower side scatter and generally higher green fluorescence than Population 2 (*EhV*), which are considered to be *Emiliania huxleyi*-specific viruses (Jacquet et al. 2002, Wilson et al. 2002). VLP1 were more numerous than *EhV* and increased in abundance from an average on 9 June of  $1.37 \times 10^6 \text{ ml}^{-1}$  to  $4.61 \times 10^6 \text{ ml}^{-1}$  by 13 June (Fig. 5). In contrast, the abundance of *EhV* remained relatively constant at between 0.3 and  $1.1 \times 10^6 \text{ ml}^{-1}$  during this early period of the mesocosm experiment (Fig. 5).

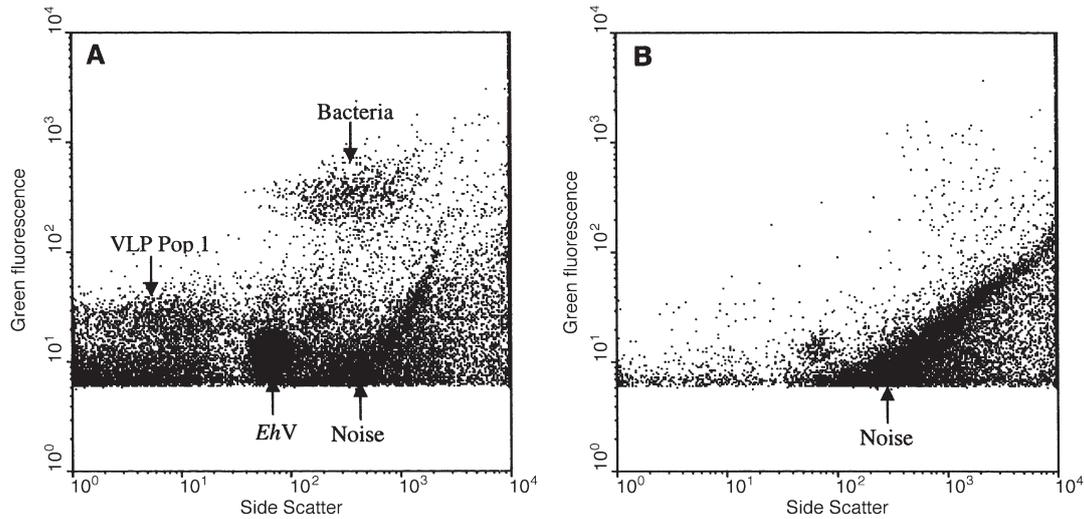


Fig. 4. Flow cytometric dot plots of the diluents produced by (A) 0.2 μm filtration and (B) 10 kDa filtration. 10 kDa filtration removed the virus-like-particle populations present in 0.2 μm filtrate. The flow cytometric signatures of the designated VLP Population 1 (VLP1) and *Emiliania huxleyi*-specific viruses (*EhV*) are indicated in (A)

**Dilution experiments and phytoplankton growth and mortality**

AFC enumeration of VLPs in the 0.2 μm and 10 kDa filtrates used in the dilution experiments indicated that viruses passed through the 0.2 μm filter but were retained by 10 kDa filtration (Fig. 4). The distribution of VLP1 abundance over the dilution series confirmed that the combination of 10 kDa filtered mesocosm water with unfiltered mesocosm water successfully produced a gradient in VLP1 concentration (Fig. 6). A similar gradient occurred with the *EhV* population in the 10 kDa dilution series. By contrast, the 0.2 μm filter

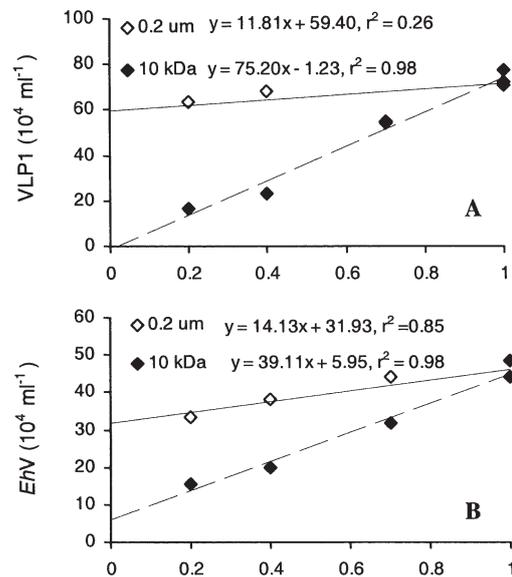


Fig. 6. VLP concentration against fraction of whole water for a dilution series set up with 0.2 μm and 10 kDa diluent for (A) VLP Population 1 and (B) *EhV* Population 2

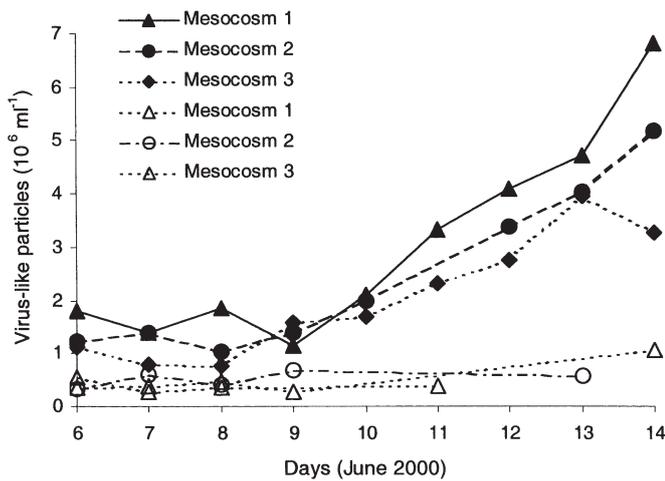


Fig. 5. Concentrations of viral populations VLP1 (black symbols) and *EhV* (white symbols) between 6 and 14 June in the 3 mesocosm bags

appeared to retain very few of the VLP1 population; therefore no significant gradient was created in the 0.2 μm dilution series (Fig. 6). The *EhV* population were retained to an extent by the 0.2 μm filter, possibly because they are larger, and a significant, although considerably lower, gradient in abundance was created in the 0.2 μm dilution series (Fig. 6). These results, particularly for the VLP1 population, appeared to validate the experimental design in which dilution incubations involving a gradient of microzooplankton

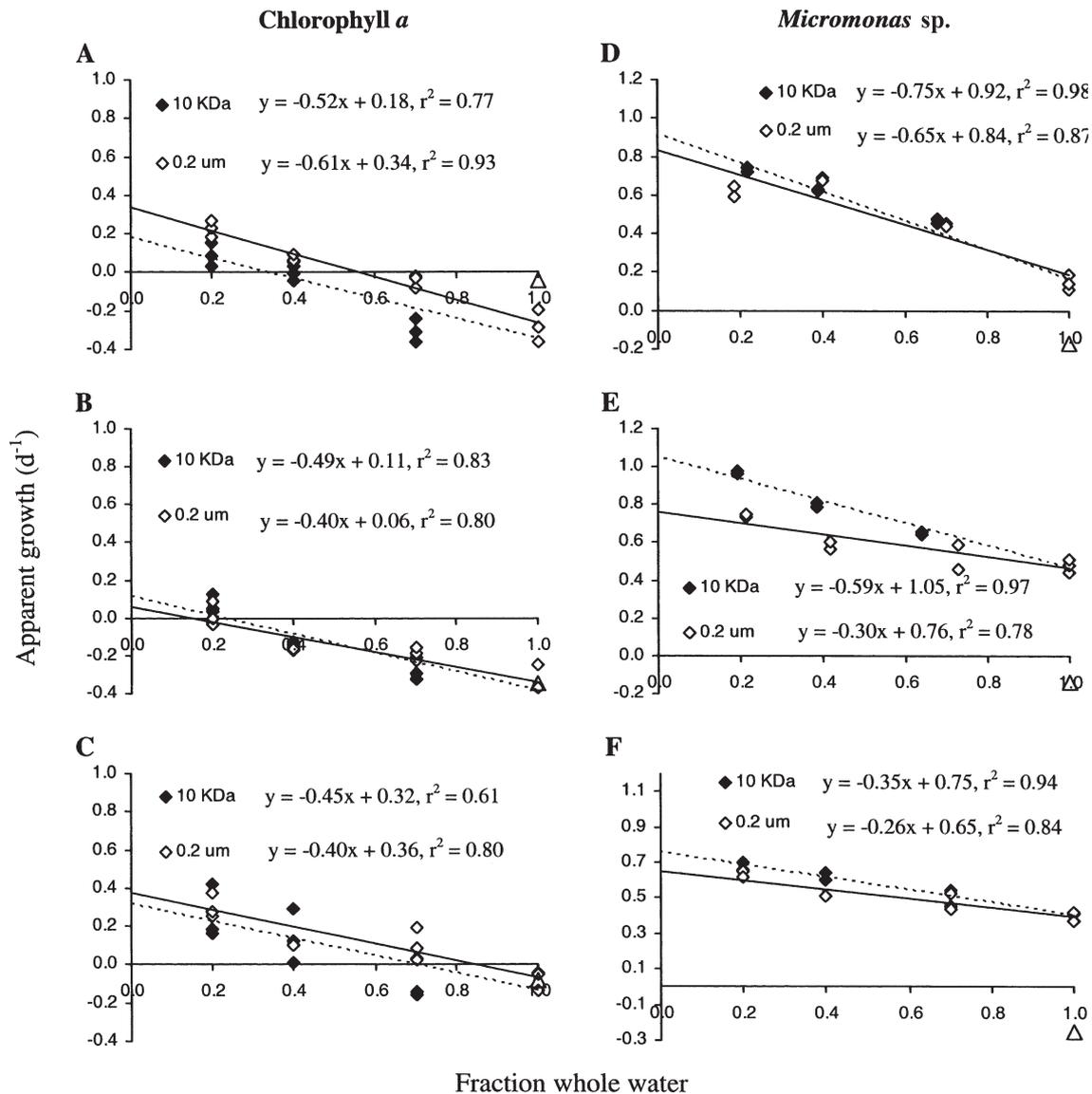


Fig. 7. Plots of apparent growth rate versus fraction of whole water in the parallel dilution experiments for chlorophyll *a* (A to C), and the *Micromonas* spp. (D to F). Plots (A) and (D) are derived from the experiments conducted using mesocosm 1 on 10 June, (B) and (E) using mesocosm 2 on 11 June and (C) and (F) using mesocosm 3 on 12 June. Equations describing the Model 1 linear regression for each experiment are shown and are included in Table 1. Standard errors for each of the regression coefficients are given in Table 1. The *in situ* net growth rates of chlorophyll *a* (A to C) and *Micromonas* spp. (D to F) determined in each mesocosm bag over the same 24 h period are shown as triangles

abundance/grazing pressure and virus concentration (10 kDa diluent) and a gradient of only grazing pressure (0.2  $\mu\text{m}$  diluent) were to be conducted in parallel.

Due to the experimental design, in which the level of dilution was controlled by the experimenter and assumed to be a proxy of the relative grazing and viral activity, Model 1 regression analysis was applied to both 0.2  $\mu\text{m}$  and 10 kDa dilution experiments. In the 3 dilution experiments conducted on 10, 11 and 12 June highly significant relationships ( $p \leq 0.001$ ) occurred

between the level of dilution and apparent growth rates of chlorophyll *a* and *Micromonas* spp. in both the 0.2  $\mu\text{m}$  and 10 kDa dilution series (Fig. 7, Table 1). Instantaneous growth rates of chlorophyll *a* and *Micromonas* spp. calculated from the dilution experiments were adjusted to reflect *in situ* rates in the mesocosm bags (Landry et al. 1995). The instantaneous growth rate determined from the dilution experiment ( $\mu$ ) was 'corrected' ( $\mu_c$ ) to an *in situ* rate by addition or subtraction of the difference between the average apparent

Table 1. Results of the parallel dilution experiments conducted on 10 and 12 June for (A) chlorophyll *a* and (B) *Micromonas* spp. Time averaged chlorophyll *a* concentration and *Micromonas* spp. abundance (<C>) in undiluted incubations are shown. Phytoplankton specific growth rates in the incubation bottles ( $\mu$ ) and mortality coefficients ( $m$ ) with respective standard errors (SE) were derived from the regression analysis of dilution plots (Fig 6). The specific growth rates were corrected ( $\mu_c$ ) to the *in situ* values measured in the respective mesocosm bags when necessary. The significance (p) of the regression analyses (ANOVA) and the significance between the slopes of the regressions of the 0.2  $\mu\text{m}$  and 10 kDa dilution series determined using an *F*-test (Sokal & Rohlf 1995) are shown

<b>(A) Chlorophyll a</b>									
Date (June)	Diluent	<C> ( $\mu\text{g l}^{-1}$ )	$\mu$ ( $\text{d}^{-1}$ )	SE	$\mu_c$ ( $\text{d}^{-1}$ )	$m$ ( $\text{d}^{-1}$ )	SE	Regression (p)	Regression slopes (p)
10	0.2 $\mu\text{m}$	3.17	0.34	0.03	0.59	-0.61	0.05	1.80E-04	
	10 kDa	3.17	0.18	0.06	0.43	-0.52	0.09	3.68E-07	0.48
11	0.2 $\mu\text{m}$	3.30	0.06	0.04	0.10	-0.40	0.06	7.38E-05	
	10 kDa	3.30	0.11	0.05	0.07	-0.50	0.08	5.97E-05	0.46
12	0.2 $\mu\text{m}$	3.37	0.36	0.05	0.28	-0.40	0.07	2.76E-03	
	10 kDa	3.37	0.32	0.07	0.32	-0.45	0.11	4.36E-04	0.71
<b>(B) <i>Micromonas</i> spp.</b>									
Date (June)	Diluent	<C> (cells $\text{ml}^{-1}$ )	$\mu$ ( $\text{d}^{-1}$ )	SE	$\mu_c$ ( $\text{d}^{-1}$ )	$m$ ( $\text{d}^{-1}$ )	SE	Regression (p)	Regression slopes (p)
10	0.2 $\mu\text{m}$	100400	0.84	0.07	0.52	-0.65	0.10	2.66E-04	
	10 kDa	100400	0.92	0.03	0.60	-0.75	0.04	5.69E-07	0.083
11	0.2 $\mu\text{m}$	73400	0.76	0.07	0.19	-0.30	0.06	1.69E-03	
	10 kDa	73400	1.05	0.03	0.41	-0.59	0.04	9.24E-07	0.001
12	0.2 $\mu\text{m}$	65500	0.65	0.03	0.00	-0.26	0.04	1.09E-03	
	10 kDa	65500	0.76	0.02	0.10	-0.35	0.03	6.21E-05	0.093

Table 2. Impact of grazing and viral mortality on production of the *Micromonas* spp. populations on 10, 11, and 12 June. Coefficients of growth, grazing and viral mortality are derived from the analysis of dilution experiments (Fig. 6, Table 1). Specific growth rate was calculated from the 10 kDa dilution series in each case and corrected to *in situ* values (Table 1). Note that the slope of regressions between 0.2  $\mu\text{m}$  and 10 kDa dilution series were significant different at only the  $p < 0.10$  level on 10, 11, and 12 June and therefore estimates of viral mortality on those dates carry the same level of significance. Potential production =  $\mu_c \times \langle C \rangle$ ; production grazed =  $m_g \times \langle C \rangle$ ; production lysed =  $m_v \times \langle C \rangle$

Date (June)	Specific growth rate ( $\mu_c$ , $\text{d}^{-1}$ )	Grazing mortality ( $m_g$ , $\text{d}^{-1}$ )	Viral mortality ( $m_v$ , $\text{d}^{-1}$ )	Potential production (cell $\text{ml}^{-1} \text{d}^{-1}$ )	Production grazed (cell $\text{ml}^{-1} \text{d}^{-1}$ )	Production lysed (cell $\text{ml}^{-1} \text{d}^{-1}$ )	Turnover due to grazing (% $\text{d}^{-1}$ )	Turnover due to viruses (% $\text{d}^{-1}$ )
10	0.61	-0.65	-0.11	60700	64800	10500	48	10
11	0.41	-0.30	-0.29	30200	21800	21300	26	25
12	0.10	-0.26	-0.10	6600	16700	6400	23	9

growth rate ( $k$ ) in the 3 undiluted incubation bottles and the net growth rate in the appropriate mesocosm bag over the same 24 h period. No adjustment was made when the *in situ* net growth rate was not significantly different ( $p < 0.05$ ) from the apparent growth rate in undiluted incubation bottles.

The net growth rate for chlorophyll *a* in undiluted incubations was similar to that observed in each of the mesocosm bags on the respective experimental days and only required adjustment for the experiment conducted on 10 June (Table 1). Corrected instantaneous growth rates ( $\mu_c$ ) measured in standard dilution experiments (0.2  $\mu\text{m}$  diluent) decreased from 0.59 to 0.10

and 0.28  $\text{d}^{-1}$  on the 3 d (Table 1), representing 0.84, 0.09 and 0.40 doublings  $\text{d}^{-1}$ , respectively. Rates of phytoplankton mortality due to microzooplankton grazing ( $m_g$ ), calculated from standard dilution experiments, ranged from -0.61 to -0.40  $\text{d}^{-1}$  (Table 1), representing a turnover of the bulk phytoplankton communities of 45 to 33%  $\text{d}^{-1}$ . For chlorophyll *a*, the slope of regression between net growth rate and level of dilution was not significantly different ( $p = 0.46$  to 0.71) between 10 kDa and 0.2  $\mu\text{m}$  dilution series, determined by applying an *F*-test (Sokal & Rohlf 1995) (Table 1). This indicates that the gradient in viral abundance created in the 10 kDa dilution series had no

greater detectable impact on net growth rates of the bulk phytoplankton community than that created in the 0.2  $\mu\text{m}$  dilution series.

Net growth rates of *Micromonas* spp. were enhanced in the undiluted incubation bottles compared to the mesocosm bags in the 3 experiments and estimates of instantaneous growth rates were adjusted to reflect this (Table 1). The adjusted, instantaneous growth rates ( $\mu\text{c}$ ) in the standard dilution experiments decreased from 0.52 to 0.00  $\text{d}^{-1}$  over the 3 d (Table 1), representing a decrease in doubling time from 0.75 to 0.00 doublings  $\text{d}^{-1}$ . The coefficients of grazing, determined from the 0.2  $\mu\text{m}$  dilution series, indicate active consumption of *Micromonas* spp. by microzooplankton grazers (Table 1). This represents a similar rate of turnover to the bulk phytoplankton community, decreasing over the 3 experimental d from 48%  $\text{d}^{-1}$  of the standing stock on 10 June to 25%  $\text{d}^{-1}$  on 12 June.

The *in situ* viral abundance in the incubations diluted with 0.2  $\mu\text{m}$  filtered water appeared to decrease the net growth rates of *Micromonas* spp. compared to 10 kDa incubations (Fig. 7). The significance of the *F*-tests for the difference between regression coefficients of mortality between 0.2  $\mu\text{m}$  and 10 kDa dilution series were 0.083, 0.001 and 0.093 on 10, 11 and 12 June respectively (Table 1). This indicates that although differences in net growth rate occurred between the dilution series on each date, an estimate of viral mortality of phytoplankton at a level of significance of  $p < 0.05$  can only be calculated from the experiment on 11 June. On the 2 other dates, estimates of viral mortality rates are only significant at  $p < 0.10$ . From the difference in slope between 10 kDa and 0.2  $\mu\text{m}$  dilution series, estimates of viral mortality amounted to a turnover of the *Micromonas* spp. standing stocks of 10, 25 and 9%  $\text{d}^{-1}$  on 10, 11 and 12 June, respectively (Table 2). The combined grazing- and viral-induced mortality of *Micromonas* spp. exceeded 100% of the daily potential production, especially on 12 June (Table 2). This was consistent with declining *Micromonas* spp. abundance in the mesocosm bags over the same period (Fig. 3). The results also suggest that estimates of the instantaneous growth of the *Micromonas* spp. populations determined from the 0.2  $\mu\text{m}$  dilution series may have been underestimated by 0.08, 0.29 and 0.10  $\text{d}^{-1}$  on 10, 11 and 12 June, respectively, compared to the 10 kDa series (Table 1).

The similar trend in *Micromonas* spp. abundance in the 3 mesocosm bags over the course of the study makes it possible to estimate the relative impact of grazing and viral mortality on population dynamics (Fig. 8). The average abundance of the *Micromonas* spp. populations in the 3 bags on each day was used to determine the population abundance. The potential abundance in the absence of grazing and viral lysis

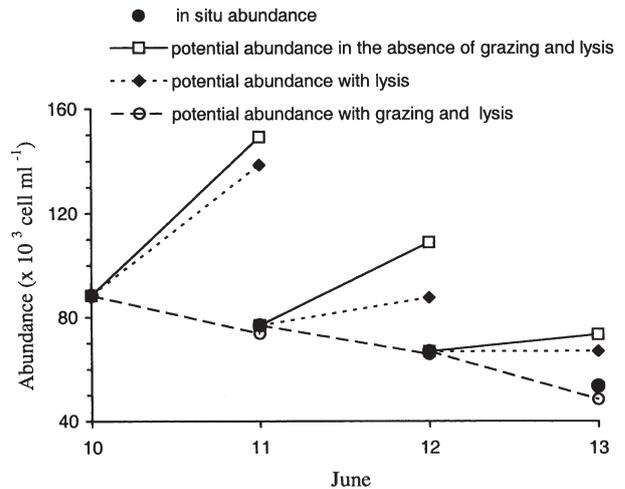


Fig. 8. Diagrammatic interpretation of the role of microzooplankton grazing and viral mortality in the decline of the *Micromonas* spp. populations in the mesocosm bags between 10 and 13 June. Average abundance in the mesocosm bags (*in situ* abundance) on each date together with the potential abundance achieved after each day and impact of viral mortality and grazing on that production (Table 2). These estimates carry with them the levels of significance given in Table 1 for each regression analysis and for the differences between the slopes for each dilution series

was calculated from adjusted instantaneous growth rates on each date (Table 2). As standing stocks and instantaneous growth rates dropped between 10 and 12 June (Table 2) the potential daily increase in abundance of the *Micromonas* spp. decreased. The contribution of viral lysis to the loss of potential production is illustrated by the potential abundance only if viral mortality losses are considered. Grazing accounted for the bulk of the phytoplankton mortality but viral lysis played a substantial role on 11 June, causing 50% of the loss of *Micromonas* spp. production.

## DISCUSSION

One of the least studied areas of marine algal virus ecology is the assessment of the quantitative importance of viruses in controlling specific host species. A major obstacle is the lack of a suitable, straightforward technique to determine virus mortality rates. The application of the dilution technique used in the present study provides a realistic explanation of the demise of the *Micromonas* spp. populations which developed within the mesocosm bags. The study illustrates that a combination of microzooplankton grazing and viral lysis exceeded the potential production of *Micromonas* spp. (Fig. 8) and consequently contributed to a rapid decline in abundance. Decreasing

instantaneous growth rates and therefore potential production, possibly due to changing environmental conditions or competition for nutrients from other taxa, meant that the *Micromonas* spp. populations were unable to respond to the high levels of mortality incurred from grazing and viral lysis. By using virus-free diluent in the dilution experiments in parallel to the conventional 0.2  $\mu\text{m}$  dilution series, we calculated that following the onset of a *Micromonas* spp. population crash, up to 34% of *Micromonas* spp. production, measured on 11 June, could be lysed daily by viruses (range 10 to 34% between 10 and 12 June; Table 2). As far as we are aware, this is the first time that a direct estimate of viral mortality of phytoplankton has been obtained by using a modification of the dilution approach of Landry & Hassett (1982). Although the approach presents new insights into the relative magnitude of viral lysis of phytoplankton and microzooplankton herbivory, it is pertinent to examine the validity of the results, and the possible implications for the conventional dilution technique.

One question raised is whether viral activity impacts on conventional dilution-based estimates of phytoplankton growth and mortality due to grazing. The flow cytometric analysis indicates that the majority of viruses passed through a 0.2  $\mu\text{m}$  pore size filter but were retained by the 10 kDa filtration (Fig. 4). There was, however, a slight gradient in the abundance of the EhV population in the 0.2  $\mu\text{m}$  dilution series (Fig. 6b), and it is likely that this group contained large (140 to 200 nm) viruses known to infect *Emiliana huxleyi* (Bratbak et al. 1996, Jacquet et al. 2002, Wilson et al. 2002). Hence, they would be more likely to be retained by filtration. Virus isolates that infect *Micromonas* spp. have a smaller size range of 105 to 130 nm (Mayer & Taylor 1979, Cottrell & Suttle 1991) and it is probable that they were part of the VLP1 population which passed through the 0.2  $\mu\text{m}$  filter. However, we are unable to rule out that different (perhaps larger uncharacterised) *Micromonas* spp.-specific virus strains would also be retained on a 0.2  $\mu\text{m}$  filter. If these larger viruses caused significant phytoplankton mortality, then the level of this viral mortality would possibly alter in relation to the gradient in viral abundance between incubations in the 0.2  $\mu\text{m}$  series and may lead to an overestimate of grazing rates. If viruses that are partially retained by a 0.2  $\mu\text{m}$  filter are a common feature of planktonic communities and cause significant phytoplankton mortality, then this may have a bearing on conventional, dilution-based estimates of microzooplankton grazing rates.

More important perhaps is the demonstration that the rate of phytoplankton growth ( $\mu$ ), determined from 0.2  $\mu\text{m}$  filtered dilution incubations, provides an estimate of phytoplankton growth in the absence of graz-

ing but does not account for viral activity and, as such, may underestimate phytoplankton productivity. For instance, on 11 June, growth rates of *Micromonas* spp. in the absence of grazing and viral lysis ( $0.41 \text{ d}^{-1}$  in the 10 kDa series) were double those determined in the absence of grazing alone ( $0.19 \text{ d}^{-1}$  in the 0.2  $\mu\text{m}$  dilution series), when corrected to *in situ* rates (Table 1, Fig. 7).

Based on measurements of chlorophyll *a*, no significant differences were determined for rates of phytoplankton mortality, and hence growth rates, between 0.2  $\mu\text{m}$  and 10 kDa dilution series (Table 1). This may reflect variable growth and mortality rates amongst the heterogeneous phytoplankton community in the 3 mesocosm bags. The *Micromonas* spp. population may have contributed between 20 and 48% of the total chlorophyll *a* over the 6 d (see 'Results'). It is therefore not surprising that the significant differences between dilution series observed for the *Micromonas* spp. populations were not observed in the chlorophyll *a* analyses.

It has become standard practice to amend incubations with additional nutrients in conventional dilution experiments and to subsequently adjust phytoplankton growth rates to *in situ* values (Landry et al. 1995). As the mesocosm bags received a daily addition of nutrients, further nutrients were not added to the dilution incubations in the present study. Net growth rates of the phytoplankton in the undiluted incubations generally exceeded those in the mesocosm bags (Fig. 7) indicating that nutrients were replete. In low nutrient-containing waters of the Gulf of Mexico, the growth rate of *Synechococcus* sp. was lower in incubations that were diluted with virus-free 10 kDa diluent compared to incubations diluted with 0.2  $\mu\text{m}$  diluent (Suttle et al. 1996, Suttle 2000). The authors suggested that *Synechococcus* growth was stimulated by enhanced recycling of nutrients due to viral lysis of hosts in seawater containing viruses (0.2  $\mu\text{m}$  diluent) compared to the virus-free water. If the modified dilution approach of the present study is applied to nutrient deplete waters then nutrient additions to both series of incubations may be required.

*Micromonas* spp. are ubiquitous and abundant components of the world's oceans (e.g. Manton & Parke 1960, Thronsen & Kristiansen 1991, Ypma & Thronsen 1996) and were the first marine microalgae against which viruses were isolated (Mayer & Taylor 1979, Waters & Chan 1982). Since their initial discovery, several studies have focused on the dynamics of virus/*Micromonas* spp. in the natural environment, revealing that the viruses which infect *Micromonas* spp. are equally ubiquitous and abundant (Cottrell & Suttle 1995a, Sahlsten 1998, Sahlsten & Karlson 1998, Zingone et al. 1999). In addition, these specific viruses are

known to be genetically diverse (Cottrell & Suttle 1991, 1995b, Chen & Suttle 1995, 1996, Chen et al. 1996). Cottrell & Suttle (1995a) calculated that from 2 to 10% of the *Micromonas* spp. population was lysed per day in inshore waters of the Gulf of Mexico. They suggested that if potential variations in adsorption coefficients were considered, high growth rates of the host (as observed in the present study, Table 1) could support infection rates as high as 50% d<sup>-1</sup> of the standing stock. Therefore, the virus-induced mortality rates calculated in the present study are within the range expected for populations of *Micromonas* spp. Cottrell & Suttle (1995a) concluded that *Micromonas* spp.-specific viruses maintained a stable coexistence with their host rather than causing a rapid decline. Similarly, Zingone et al. (1999) noted that viral infection was not correlated to the decline of *Micromonas* spp. populations in the Mediterranean Sea. The decline of the *Micromonas* spp. population in the present study may have been linked to an increasing abundance of VLP1 (Fig. 5). However, further studies are required to ascertain whether the VLP1 population consists of *Micromonas* spp.-specific viruses. Although viruses played an important part in the demise of the *Micromonas* spp. bloom in the current study, a combination of intense grazing pressure (Table 2), virus infection and possibly competition for nutrients from other phytoplankton eventually dominated by *Emiliana huxleyi* (Jacquet et al. 2002), all contributed to the phytoplankton succession observed in the mesocosms.

A wide variety of approaches have been used to calculate virus production rates from which estimates of viral-induced mortality of phytoplankton can be extrapolated (reviewed in Suttle 2000, Wommack & Colwell 2000). Rates for virus-induced phytoplankton mortality typically range from 3 to 20% d<sup>-1</sup> of the standing stock. However, estimates from 0 to over 100% d<sup>-1</sup> have been reported (Wommack & Colwell 2000). Several alternative approaches have involved manipulation of virus/host concentrations. In contrast to the dilution approach, some earlier studies added increasing amounts of concentrated virus communities to natural seawater samples (reviewed in Suttle 1994). This approach allowed an estimate of the photosynthetic rate in the absence of viruses to be obtained from the y-intercept of a linear regression calculated from the initial slope of a photosynthesis suppression curve. Using this method predicted rates of primary productivity were enhanced by 1.2 to 3.7% in the absence of viruses (Suttle 1994). Recently, using an alternative 'dilution' approach, Hewson et al. (2001) concentrated the bacteria/phytoplankton fraction by retention on a 0.22 µm filter then diluted the cellular concentrate with virus-free seawater. Virus production was then calculated by measuring the rate of increase of virus parti-

cles over time. Wilhelm et al. (2002) used a modification of this approach, in which the >0.2 µm fraction was kept in suspension whilst gradually being diluted with virus-free water, to estimate virus production rates and extrapolate lytic rates of bacteria.

Results from the present study reveal that a dilution approach can be adapted to directly determine virus-induced mortality rates of specific phytoplankton. However, the specificity of viral infection and variety of viral infection cycles adds increased complexity to determining rates of viral mortality of phytoplankton. In particular, the duration of the latent period, the time between viral infection and host lysis, is likely to be a critical factor in determining the detection of viral mortality by the dilution approach. Viral mortality may be most easily quantified if the latent period falls within the duration of the dilution incubations, typically 24 h. This is particularly the case if cell counts are used to determine net growth rates in the incubations. Hence, the technique is likely to favour lytic infections of phytoplankton species with high growth rates. In future, it may be advantageous to tailor incubation times to the specific virus propagation period of the phytoplankton of interest, or to sequentially sub-sample incubations over an extended period.

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